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Miracidal Hatching in the Diagnosis of Bilharziasis

BY

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Introduction.

Dr. Friedrick Fulleborn (1921) was the first worker to describe the hatching technique for the emergence of schistosome miracidia. Dr. Fulleborn (1866-1933) qualified in medicine at Berlin University and worked as Virchow's voluntary assistant. He first made his mark as a zoologist, but became associated with the Institute of Tropical Medicine at Hamburg in 1901 after spending four years with the German Army. He later became the director, and was an outstanding figure in German tropical medicine. He did pioneer work in the study of *Schistosoma, Ancylostoma, Filaria* and *Strongyloides* — much of which belong to the classics.

Essentially Fulleborn's method for the preparation and hatching of stool specimens consisted of three to four washings of the stool specimen in a 3-4 per cent. sodium chloride solution. The specimen was allowed to sediment for five minutes in a conical flask between each washing. Samples were pipetted out on to a microscope slide for examination. Warm water (45°-50°C) was added to the remaining sediment in the conical flask and placed in the light. Very soon miracidia hatched out and these could be seen with a magnifying glass.

It is rather surprising that little work has been done to develop Fulleborn's method.

For the past twenty-five years we have been using an improved technique, and here we found this to be of tremendous value in incidence and prevalence surveys and drug trials.

Fulleborn (*op. cit.*) said then that the diagnosis of bilharziasis could be carried out without the use of a microscope and we fully endorse his statement.

The following techniques are currently employed at this laboratory in diagnosing bilharziasis.

Method.

Apparatus and Reagent:

Meeser *et al* (1948) described a simple apparatus which is used in the hatching of stool and urine specimens. Standard 15 ml centrifuge tubes are used. The sectional drawing and diagrams are in the original article. The examination rack is made of wood painted matt

black, and consists of two parts — the examination rack itself, and the hand-lens holder or miracidiascope. The rack is 32 cm long and allows ten centrifuge tubes to be examined at one time. The tubes are held in position, centre 2,5 cm apart, by a series of holes in the back plate, the tip of each tube resting against a serration in a strip of plywood along the front edge of the base of the rack. Each tube will lie at an angle of 40° to the horizontal and be in the correct optical angle for viewing with the hand lens.

The hand lens should be about 2,5 cm in diameter, and have a focal length of 6,3 cm. The handlens holder should hold the hand lens at an angle of 40°, 6,3 cm away from the centre of the tubes in the rack. The reagent used is added to the deposit in the centrifuge tube and is termed "hatching water".

It has been found from experience that care must be taken when preparing hatching water. Ordinary tap water is not suitable — probably due to the chlorine present in this water. Distilled water is also unsuitable. We have found that tap water which has been de-chlorinated by fish is suitable. However, the best source of water is that which has been filtered at the waterworks and taken before any chlorine has been added. In both cases the water must then be subjected to heat of 56°C to remove any infusoria which might be mistaken for miracidia.

Collection, preparation and examination of urine specimens:

Urine specimens are collected in 110 ml glass jars which have bakelite screw-caps.

1. The patient is given a glass jar and asked to pass terminal urine into it between 1000 and 1400 hours (Weber *et al*, 1967). All his terminal urine must be expressed into the jar.
2. Upon reaching the laboratory the cap of the bottle is removed and the bottle allowed to stand for 30 minutes. The supernatant urine is then drawn off with a water vacuum pump, making sure that the sediment is not disturbed, and leaving behind 10-15 ml of urine.
3. The urine in the glass jar is then agitated and its contents emptied into a standard 15 ml centrifuge tube then placed into a centrifuge and spun at 1 000 rpm for 90 seconds.
4. The supernatant urine is again removed with the water vacuum pump — this time leaving behind 0,5 ml. The specimen is now ready for microscopic and macroscopic examination.

Examination of urine specimens under the microscope:

1. The centrifuge tube is placed into the palm of one hand and flicked with the finger of the other hand to re-suspend the schistosome ova — if present in the tube.
2. Using a Pasteur pipette 0,05 ml of urine is drawn up, placed on a microscope slide, and a cover-glass placed on top.
3. The slide is then placed on a microscope and, with a scanner objective x4, all the ova under the cover-glass are counted and the sum multiplied by ten — thus giving the estimated total number of eggs present in the specimen. The remaining nine-tenths of the urine is put up for hatching.

The hatching of urine specimens:

Hatching water is added to the remaining deposit of urine in the centrifuge tubes and the tubes placed into the examination racks — as previously described.

The racks are placed facing into sunlight. The temperature of the tubes is brought up to about 38°C then the racks are removed and placed in the shade so that the convection currents may settle. The specimens are now ready for viewing with a miracidiascope. This process must be repeated at each observation.

The racks are then turned so that daylight or light from an artificial source streams down the length of the centrifuge tube illuminating the contents of the tube against the dark background of the rack. A good procedure when viewing is to give the tube a quarter turn; this tends to activate the miracidia and remove any urine and stool deposit from the back of the tube and out of the direct line of sight. Miracidia have a distinctive swimming movement; they swim in a direct line, zig-zagging across the field of vision. Occasionally one will be seen doing a cartwheel motion; this happens when the miracidium has not freed itself completely from the shell.

When conducting large surveys, set times for examination must be adhered to, the times which are most convenient being 1000, 1200 and 1500 hours. Urine specimens must be examined quickly if good hatching is to be obtained.

During the hotter months of the year little difficulty is encountered in warming up the tubes; the problem is to prevent them from becoming too hot. During winter and on rainy days, warming the tube presents a problem but we have overcome this by using a heating cabinet with bright artificial light — though the results are not as good as exposure to direct sunlight.

Hatching of urine is not as straight-forward as that of stool specimens. Several factors prevent good hatches. It has been found that fresh specimens of urine gave a much better result than those which were put up for hatching 24 hours after they had been passed. The process of getting rid of the red blood cells in certain specimens is time consuming, and in some cases impossible, thus it is essential to wash them several times in physiological saline. The tube is then centrifuged at 1 000 rpm for 90 seconds. Decant by gently tipping the tube upside down. Repeat the process then add the hatching water to the remaining deposit.

The following standard was used to estimate the number of miracidia:—

0 — no hatch
+ — 1-8 miracidia
2+ — 9-20 miracidia
3+ — 21-40 miracidia
4+ — 41-80 miracidia
5+ — large numbers of miracidia

Collection, preparation and examination of stool specimens:

1. The patient is given a glass jar containing 40 ml double strength physiological saline (18g NaCl per litre) and a small, flat wooden spatula and asked to pass a stool either on to a wad of toilet paper or on to the ground, then with the aid of the spatula, to place a piece of stool the size of a walnut into the glass jar containing the saline solution.
2. The specimen is then given a good shake.
3. When the specimen arrives at the laboratory it is given another vigorous shake then sieved through a fine, phosphobronze 100 mesh wire sieve into a conical urine sedimentation flask. The debris on the sieve is then washed with physiological saline (9,0g NaCl per litre) from a squeeze bottle. The flask is topped up with physiological saline and allowed to sediment for 30 minutes.
4. Using a water vacuum pump, the supernatant liquid is sucked off ensuring that the deposit at the bottom of the flask is not disturbed, topped up again with physiological saline and allowed to stand for a further 30 minutes.
5. Once again use a water vacuum pump to suck off the supernatant liquid and do not disturb the deposit. If it is seen that the supernatant liquid is still turbid, a third wash is necessary. The deposit must then be swirled and the entire contents emptied into a standard 15 ml centrifuge tube, placed in a centrifuge and spun at 1 000 rpm for 90 seconds.
6. The supernatant liquid is removed with a water vacuum pump, leaving behind 0,5 ml of deposit.

The specimen is now ready for microscopic and macroscopic examination.

Examination of stool specimens under the microscope:

1. The centrifuge tube is placed into the palm of one hand and flicked with the finger of the other hand to re-suspend the schistosome ova if present.
2. Using a Pasteur pipette 0,05 ml of stool sediment is drawn up, placed on a microscope slide and a cover-glass placed on top.
3. The slide is then placed on the microscope and with a scanner objective x4 the number of eggs under the cover-glass is estimated using the following table:—

+ — being less than 5 eggs of *S. mansoni* in the specimen
2+ — up to 20 eggs on the slide
3+ — being eggs in every traverse of the cover-glass preparation
4+ — some eggs in every field, and
5+ — many eggs in every field.

If no water vacuum facilities are available the preparation of stool and urine specimens can be carried out by manual tipping of the sedimentation flask, urine bottle and centrifuge tube to remove the supernatant material. This is done by allowing the supernatant liquid to run slowly and steadily to leave in the container not more than the amount of sediment required for the next stage of the preparation.

The water vacuum pump allows large numbers of specimens to be processed speedily Clarke (1965) stated "that this method of sedimentation examination, although satisfactory for prevalence surveys, was not fully satisfactory for estimating egg production because of errors inherent in the technique."

The hatching of stool specimens:

The same procedure applies to stools as that described for urine specimens. Stools do not have to be put up for hatching as quickly as urine (Blair *et al*: 1969).

It has been the practice in this laboratory to put all the stool specimens up for hatching for a period of at least 48 hours or six examinations, but we found that 94 per cent. of the total 3 867 specimens that were positive came up on the first day. The best hatch was obtained at mid-day of the first day, but many specimens continued to hatch throughout the six observations. On the second day the stool specimen should be well agitated and topped up with hatching water if necessary, to compensate for loss through evaporation.

From our survey records, 1968-1973 stool survey results were selected at random to show the number of positives that could be missed by employing only microscopic examination. Out of a total of 2 887 stool specimens selected, 1 204 were positive both on microscopic and macroscopic examination; 1 057 were completely negative; 150 positive microscopically and negative macroscopically; 476 negative microscopically and positive macroscopically. Thus 16 per cent. of all specimens examined would have been missed had the specimens not been subjected to hatching. Likewise, if all the specimens were subjected to hatching, only 5 per cent. or 150 out of 2 887 would not be detected.

Discussion and Conclusion.

Fulleborn first described the preparation and hatching of stool specimens, yet surprisingly little work has been done since to try to develop this technique. The reason may be the fact that the article was printed in German. His method of hatching has tremendous advantages which very few workers seem to appreciate. In public health programmes a patient who is passing ova which will not hatch is not a source of infection.

During examination of a patient, where microscopic examination has yielded negative results, hatching will reveal a very light infection, if present.

In drug trials hatching plays an important part in assessing the efficacy of the drug. This applies particularly to urine specimens where the patient continues to pass dead ova, but no miracidial hatch was obtained after successful therapy (Weber, *et al.* 1969).

Microscopic examination requires skilled labour and expensive equipment, e.g., a microscope. The technical knowledge required for macroscopic examination for the hatching of miracidia is quickly attained and requires little technical skill.

Our results show that it is not necessary to hatch for a period of 48 hours unless carrying out the follow-up of drug trials or examination of special patients. Three observations during one day is sufficient, and will save time. It has been found that urine specimens should not be kept for hatching longer than one day.

Rural hospitals, mission hospitals and clinics would be able to carry out this type of examination for bilharziasis because it is inexpensive and does not require skilled technical knowledge.

Summary.

Fulleborn 51 years ago described this technique, yet remarkably little use has been made of it in diagnosis. His method — with improvements — for examining large numbers of specimens has been in use in this laboratory for over 25 years. It has been of special value in survey work in Rhodesia in endemic areas. The method has proved of particular value in drug trials. Miracidial hatching used alone could extend efficient diagnosis of bilharziasis to smaller medical units with no laboratory facilities.

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